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Article

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Abstract

Objective: Previous studies indicated a difference in crosstalk between canonical WNT pathway and nuclear factor-κB (NFκB) signaling in human and animal chondrocytes. To assess whether the differences found were dependent on cell types used, we tested the effect of WNT modulation on NFκB signaling in human primary articular chondrocytes in comparison with the immortalized human costal chondrocyte cell line C20/A4. *Design:* We used gene expression analysis to study the effect of WNT modulation on IL1β-induced matrix metalloproteinase (MMP) expression as well as on WNT and NFκB target gene expression. In addition, we tested the involvement of RelA and TCF4 on activation of the WNT and NFκB pathway by TCF/LEF and NFκB reporter experiments, respectively. *Results:* We found an inhibitory effect of both induction and inhibition of WNT signaling on IL1β-induced MMP mRNA expression in primary chondrocytes, whereas WNT modulation did not affect MMP expression in C20/A4 cells. Furthermore, TCF/LEF and NFκB reporter activation and WNT and NFκB target gene expression were regulated differentially by TCF4 and RelA in a cell type–dependent manner. Additionally, we found significantly higher mRNA and protein expression of TCF4 and RelA in C20/A4 cells in comparison with primary chondrocytes. *Conclusions:* We conclude that WNT modulation of NFκB is, at least in part, cell type dependent and that the observed differences are likely because of impaired sensitivity of the NFκB pathway in C20/A4 cells to modulations in WNT signaling. This might be caused by higher basal levels of TCF4 and RelA in C20/A4 cells compared to primary chondrocytes.

Keywords
C20A4, chondrocyte, crosstalk, NFκB, WNT signaling

Introduction

The balance between the breakdown and synthesis of extracellular matrix is crucial for the integrity of the structure and function of articular cartilage. This homeostasis is maintained by catabolic and anabolic processes that are regulated by chondrocytes and other cells in articular joints. Interleukin (IL)-1β was found to be involved in disturbed homeostasis, mainly due to increased expression and activity of matrix metalloproteinases (MMPs), leading to cartilage degeneration.[1,2] One of the major regulators of catabolic processes induced by IL1β is the nuclear factor (NF)-κB signaling pathway.[3,4] In the absence of an inflammatory signal, the p50 and p65 subunits form an inactive heterotrimer with IκBα, which resides in the cytoplasm. After activation of the pathway, IκBα is removed from the complex and p50 and p65 translocate to the nucleus to regulate gene transcription through NFκB binding sites in promoters of target genes.[5] The NFκB pathway was found to be essential for the expression of MMP1, MMP3, MMP13, and IL6, IL1β, and tumor necrosis factor (TNF)-α. In addition, inflammatory activation of the NFκB pathway can induce hypertrophic differentiation, which is involved in cartilage degenerative disease.[6]

Recently, WNT/β-catenin signaling was found in IL1β-induced cartilage degradation.[7-9] In absence of a WNT ligand, β-catenin is phosphorylated by a degradation complex and subsequently degraded by the proteasome. On

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binding of a WNT ligand to the receptor Frizzled (FZD) and its co-receptors low-density lipoprotein receptor–related protein (LRP) 5/6, the degradation complex is disassembled, resulting in accumulation of β-catenin in the cytoplasm. Subsequently, β-catenin can translocate to the nucleus to bind T-cell specific factor (TCF)-4 and lymphoid-enhancer factor (LEF)-1, thereby regulating gene expression.[10] Our group has recently shown that in human chondrocytes, canonical WNT signaling is a potent inhibitor of pro-inflammatory mediator-induced MMP expression by counteracting pro-catabolic NFκB. The inhibitory action of WNT signaling was found to be due to direct interaction of β-catenin with the NFκB pathway,[8] found in various cancer cells.[11,12] More specifically, it was shown that β-catenin interacted with RELA.[11] These observations indicated that β-catenin has an anti-catabolic effect by inhibiting MMP expression in human cartilage, whereas a pro-catabolic role of β-catenin in animal cartilage was reported.[7,13] However, β-catenin interacted with the NFκB pathway and inhibited NFκB-mediated signaling in mouse cells, suggesting that β-catenin–RELA interaction is not responsible for the species-specific effect of WNT signaling on MMP expression.[8] In contrast to human chondrocytes, induction of MMP expression by IL1β in animal chondrocytes was dependent on β-catenin.[8] In addition, our group has shown that overexpression of TCF4 in human chondrocytes, expressed higher in human osteoarthritic cartilage, induced the expression and activity of MMPs in human chondrocytes.[14] Furthermore, induction of MMP expression by TCF4 appeared to be due to the potentiating effect of TCF4 on NFκB signaling likely by direct interaction with RELA.[14]

The dissimilarity in the interaction between the WNT pathway and NFκB signaling was attributed to a species-specific effect.[8] To verify whether this effect was rather caused by differences in the cellular environment, we assessed the effects of induction and inhibition of WNT/β-catenin signaling on NFκB and related MMP expression in human primary articular chondrocytes (hCh) and the immortalized human costal chondrocyte cell line C20/A4. We found active crosstalk between the WNT pathway and NFκB signaling in hCh, which was partial or absent in C20/A4, indicating a cell-specific interaction between WNT and NFκB.

Methods

Cell Isolation and Culture

Human primary articular chondrocytes were isolated from healthy looking cartilage of the femoral condyles of patients undergoing total knee replacement surgery. Isolated cartilage was digested with collagenase type II at 37°C overnight and cells were isolated using a 100-μm cell strainer. Isolated hCh were cultured as previously described[15] and used at passage 2. Immortalized human juvenile costal chondrocytes, C20/A4 cells,[16] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The use of human biopsies was approved by the local ethical committee and all patients gave their consent for the use of their tissues.

Viral Transduction Assays

Human primary articular chondrocytes and C20/A4 were seeded at 7500 cells/cm^2 in 96-well plates (Nunc International) and cultured for 24 hours, prior to transfection with lentiviral TCF/LEF reporter and pRL-CMV control (both Promega, Madison, WI). Cells were treated with 200 ng/mL recombinant human WNT3A (high purity, R&D Systems, Minneapolis, MN), 1.0 μM GSK3β inhibitor BIO (Sigma Aldrich, St. Louis, MO) or 1.0 μM PKF115-584 (Novartis, Basel, Switzerland), a small molecule blocking binding of β-catenin to transcription factor TCF4.[17] All treatments were performed with or without addition of 10 ng/mL IL1β (R&D Systems, Minneapolis, MN). After 48 hours of stimulation, luminescence was measured using the Dual-Glo luciferase assay kit (Promega, Madison, WI).

Gene Expression Analysis

Human primary articular chondrocytes and C20/A4 were seeded at 25,000 cells/cm^2 in 24-well plates (Nunc International, Rochester, NY) and cultured for 24 hours before addition of 200 ng/mL recombinant human WNT3A (high purity, R&D Systems), 1.0 μM GSK3β inhibitor BIO or 1.0 μM PKF115-584 with or without addition of 10 ng/mL IL1β (R&D Systems). Forty-eight hours later, cells were harvested and analyzed as previously described.[15] Gene expression was normalized using B2M and expressed as fold induction compared with controls.

Western Blot

For Western blot analysis, cells were lysed using RIPA buffer (Cell Signaling Technology, Danvers, MA), supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA). Antibodies used for Western blot were mouse anti-human β-catenin (1:1000, BD Biosciences, Franklin Lakes, NJ), rabbit anti-human NFκB p65 (RELA, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human TCF4 (1:1000, Cell Signaling), and rabbit anti-mouse and goat anti-rabbit secondary antibodies (1:1000, Invitrogen, Carlsbad, CA). SuperSignal West Femto Substrate (Thermo Scientific) was used for visualization of the bands.

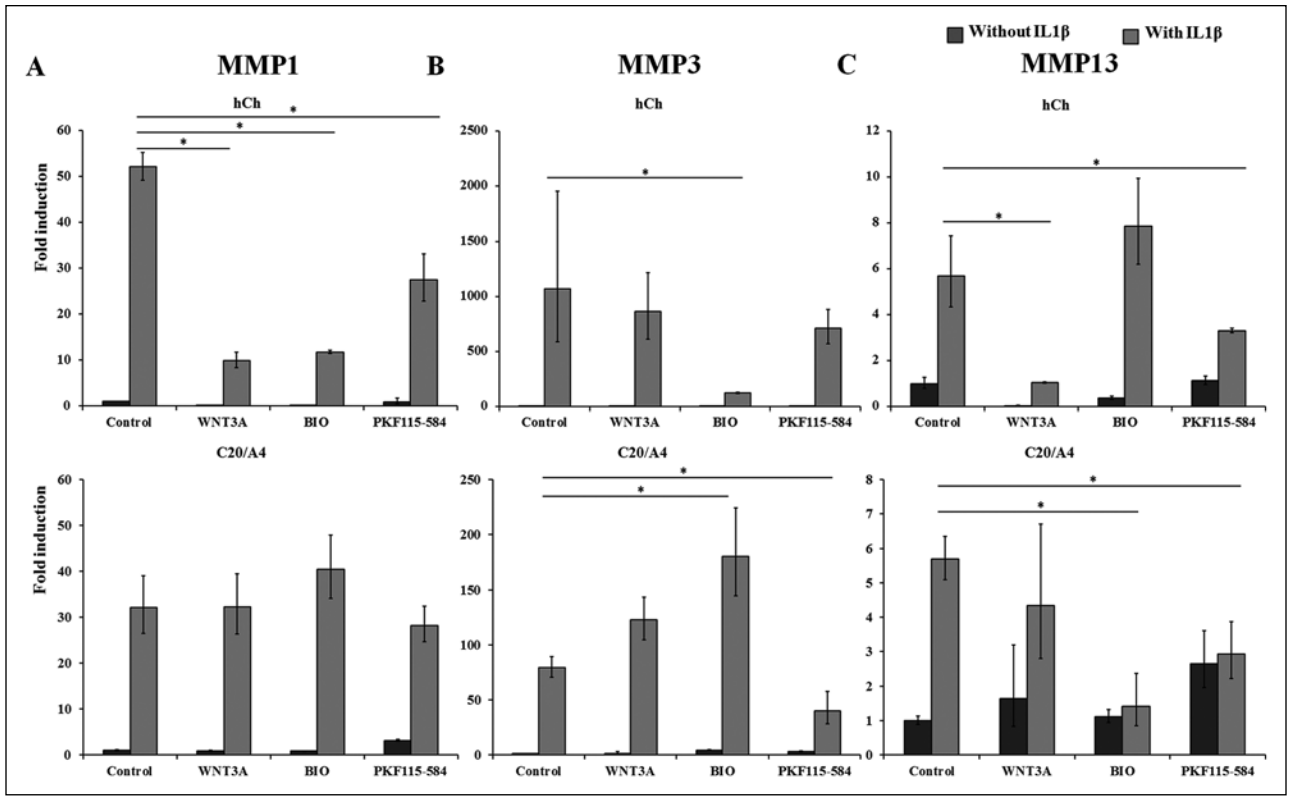

Figure 1. Distinct effects of WNT modulation in human primary articular chondrocytes (hCh) and C20/A4. (**A**) IL1β (10 ng/mL) induced expression of *MMP1* was inhibited by co-treatment with WNT3A (200 ng/mL) and BIO (1.0 μM) as well as with PKF115-584 (1.0 μM) in hCh, whereas in C20/A4, *MMP1* expression was not affected by any of the compounds. (**B**) IL1β-induced *MMP3* expression was only inhibited by BIO in hCh and by PKF115-584 in C20/A4. (**C**) The mRNA expression of *MMP13* was inhibited by WNT3A and PKF115-584 in hCh, whereas in C20/A4, both BIO and PKF115-584 blocked IL1β-induced *MMP13* expression. Expression levels were determined by qPCR after normalization for a house hold gene. Graphs are representative for 3 independent experiments and expressed as fold induction compared with controls ± 95% confidence interval.

Statistical Analysis

Results were expressed as mean values ± 95% confidence intervals (CIs) and statistical significance was tested using analysis of variance for multiple conditions or Student's *t* test for comparing 2 conditions (PASW Statistics 18).

Results

The Effect of WNT Signaling on mRNA Expression of MMPs Is Cell Type Dependent

Since basal MMP expression is relatively low in chondrocytes, cells were treated with IL1β to induce gene expression. This enabled us to study the effect of induction and inhibition of canonical WNT signaling on MMP expression. In basal conditions in hCh, neither WNT3A and BIO nor PKF115-584 affected MMP expression. IL1β potently induced *MMP1, MMP3*, and *MMP13* expression. Interestingly, both activation of canonical WNT signaling by treatment with WNT3A and BIO, as well as inhibition of canonical WNT signaling by PKF115-584, inhibited IL1β-induced *MMP1* expression. In C20/A4, *MMP1* expression was not significantly affected by activation or inhibition of WNT signaling, neither in basal conditions nor after IL1β induction (**Fig. 1A**). The mRNA expression of *MMP3* in hCh was significantly inhibited by BIO, whereas the expression was not significantly changed by WNT3A and PKF115-584. In contrast, in C20/A4, IL1β-induced *MMP3* expression was slightly enhanced by co-treatment with WNT3A and BIO, whereas PKF115-584 inhibited *MMP3* expression (**Fig. 1B**). In hCh, WNT3A and PKF115-584 repressed IL1β-induced *MMP13* expression, whereas BIO did not significantly affect *MMP13* expression. The expression of *MMP13* was inhibited by WNT3A, BIO, and PKF115-584, although not significantly changed by WNT3A in C20/A4 (**Fig. 1C**). The effect of inhibition of WNT/β-catenin signaling by PKF115-584 is similar in hCh and C20/A4. However, the inhibitory effect of WNT3A and BIO on *MMP3* expression in hCh is opposite to the stimulating effect in C20/A4. The dissimilarity of WNT3A, which acts at the receptor level, was not

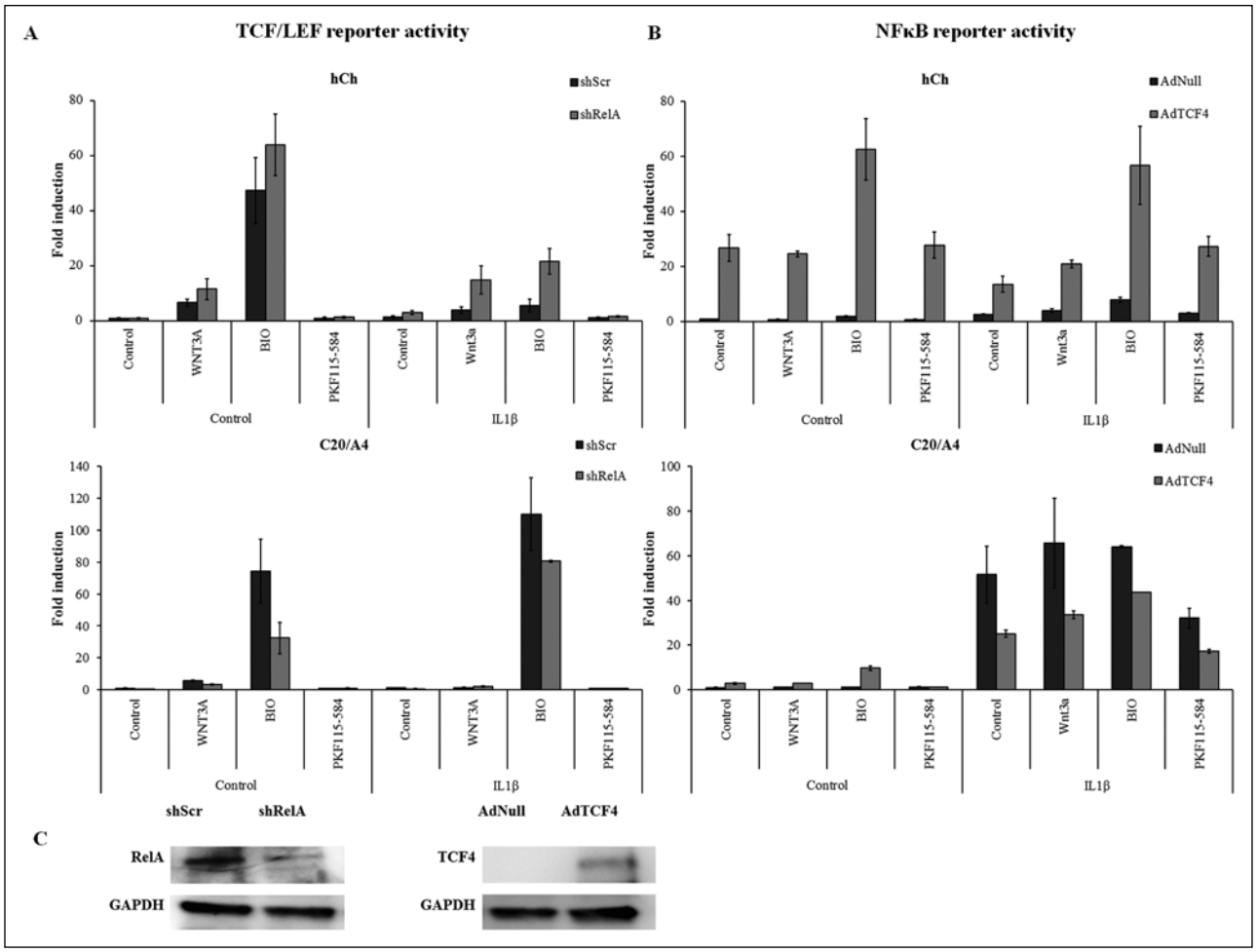

Figure 2. Overexpression of TCF4 enhances IL1β induction of NFκB reporter activity in human primary articular chondrocytes (hCh), but blocks IL1β-induced NFκB activity in C20/A4. (**A**) TCF/LEF reporter activity in hCh is upregulated by both WNT3A and BIO, which is slightly enhanced by knockdown of RELA. IL1β inhibits the induction of TCF/LEF reporter by BIO. In C20/A4, TCF/LEF reporter activity is induced by WNT3A and BIO and slightly inhibited by knockdown of RELA. (**B**) NFκB reporter activity is induced by IL1β in hCh and enhanced by WNT3A and BIO. Overexpression of TCF4 induces NFκB reporter activity in hCh. In addition, in C20/A4 NFκB reporter activity is induced by IL1β, which is inhibited by overexpression of TCF4. (**C**) Western blot analysis confirms partial downregulation of RELA protein expression by shRELA and upregulation of TCF4 by AdTCF4. Data is representative for 3 independent experiments and expressed as fold induction compared with control ± 95% confidence interval.

as pronounced as the opposing effect of BIO, which acts more downstream in the WNT/β-catenin pathway.

These findings indicate a cell type dependent effect of modulation of the WNT signaling pathway on cytokine-induced MMP mRNA expression, which might be regulated by β-catenin or at the transcriptional level. Therefore, we tested the involvement of downstream WNT effectors β-catenin and TCF4 and the NFκB mediator RELA on TCF/LEF and NFκB-promoter reporter constructs.

TCF4 Enhances IL1β-Induced NFκB Reporter Activity in hCh but Not in C20/A4

To test the involvement of the NFκB signaling mediator RELA in the WNT pathway, cells were co-transduced with a TCF/LEF reporter construct and a short hairpin (sh) RNA construct for RELA. In hCh, WNT3A and BIO induced TCF/LEF reporter activity, whereas the TCF/β-catenin inhibitor PKF115-584 did not affect the TCF/LEF reporter. The upregulation of the TCF/LEF reporter by WNT3A and BIO was slightly enhanced by the knockdown of RELA, while co-treatment with IL1β inhibited reporter activation by BIO. In C20/A4, WNT3A and BIO activated the TCF/LEF reporter, whereas reporter activity was not affected by addition of IL1β. Knockdown of RELA inhibited BIO-induced upregulation of TCF/LEF reporter activity (**Fig. 2A**). This suggests that TCF/LEF reporter activation by BIO is at least partly dependent on RELA.

To study the involvement of TCF4 in the activation of the NFκB pathway, TCF4 was adenovirally overexpressed in cells

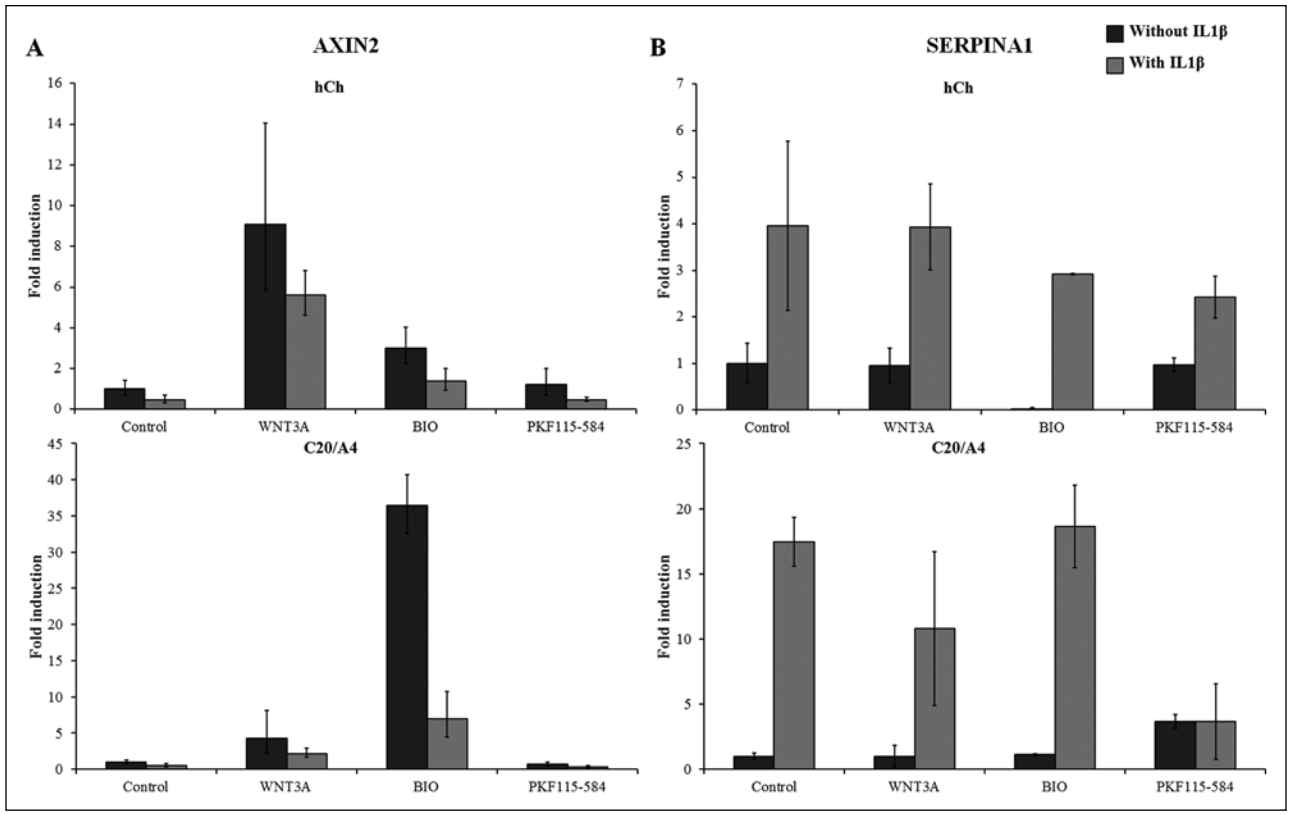

Figure 3. (**A**) In hCh, *AXIN2* expression was upregulated by both WNT3A and BIO, whereas PKF115-584 did not affect *AXIN2* expression. Addition of IL1β slightly inhibited upregulation of *AXIN2* expression by WNT3A and BIO. In C20/A4, *AXIN2* expression was induced by WNT3A and BIO, which was not significantly affected by IL1β. PKF115-584 did not affect AXIN2 expression. (**B**) *SERPINA1* expression was induced by IL1β in both hCh and C20/A4. Upregulation was inhibited by BIO and PKF115-584 in hCh, but differences were not significant. However, in C20/A4, IL1β induced *SERPINA1* expression was slightly enhanced by BIO, whereas WNT3A (not significant) and PKF115-584 inhibited IL1β-induced *SERPINA1* expression. Expression levels were determined by quantitative polymerase chain reaction (qPCR) after normalization for a house hold gene. Data are representative for 3 independent experiments and expressed as fold induction compared with control ± 95% confidence interval.

that were transduced with the NFκB promoter reporter construct. NFκB reporter activity was significantly induced by IL1β by 2.6 fold ($P = 0.03$), which was enhanced by both WNT3A and BIO in hCh. Additionally, overexpression of TCF4 strongly induced NFκB reporter activity, which was further enhanced by co-treatment with BIO. In C20/A4, the NFκB reporter was strongly induced by IL1β by 7.9 fold ($P = 0.01$), without significant effects of WNT3A, BIO, or PKF115-584. Interestingly, IL1β-induced NFκB reporter activity was inhibited by overexpression of TCF4 (**Fig. 2B**). This suggests that TCF4 is inductive for NFκB reporter activity in hCh, but inhibitory in C20/A4.

The knockdown of RELA by shRELA and the overexpression of TCF4 by AdTCF4 were confirmed by Western blot analysis (**Fig. 2C**).

Target Gene Expression Is Affected by WNT Modulation in a Cell Type–Specific Manner

To verify whether the WNT signaling pathway is equally active in hCh and C20/A4, we assessed the effects on *AXIN2* mRNA expression. In both hCh and in C20/A4, *AXIN2* mRNA expression was induced by WNT3A and BIO, however, BIO was more potent in C20/A4 than in hCh. As expected, PKF115-584 did not affect *AXIN2* expression. Co-treatment with IL1β did not significantly affect *AXIN2* expression in hCh and C20/A4, but was significantly reduced in BIO treated C20/A4 cells (**Fig. 3A**).

We next examined the effect on the established NFκB target gene *SERPINA1*. The upregulation of *SERPINA1* by IL1β was much stronger in C20/A4, when compared to hCh. IL1β induced *SERPINA1* expression was not significantly changed by BIO and PKF115-584 in hCh. In C20/A4, WNT3A and PKF115-584 inhibited *SERPINA1* expression, whereas BIO upregulated IL1β induced *SERPINA1* mRNA expression (**Fig. 3B**).

The effect of IL1β stimulation as well as the influence of WNT modulation was much stronger in C20/A4 compared with hCh. Since RELA and its interaction with TCF4 seem to be involved in IL1β-induced activity, we next assessed the expression levels of these mediators.

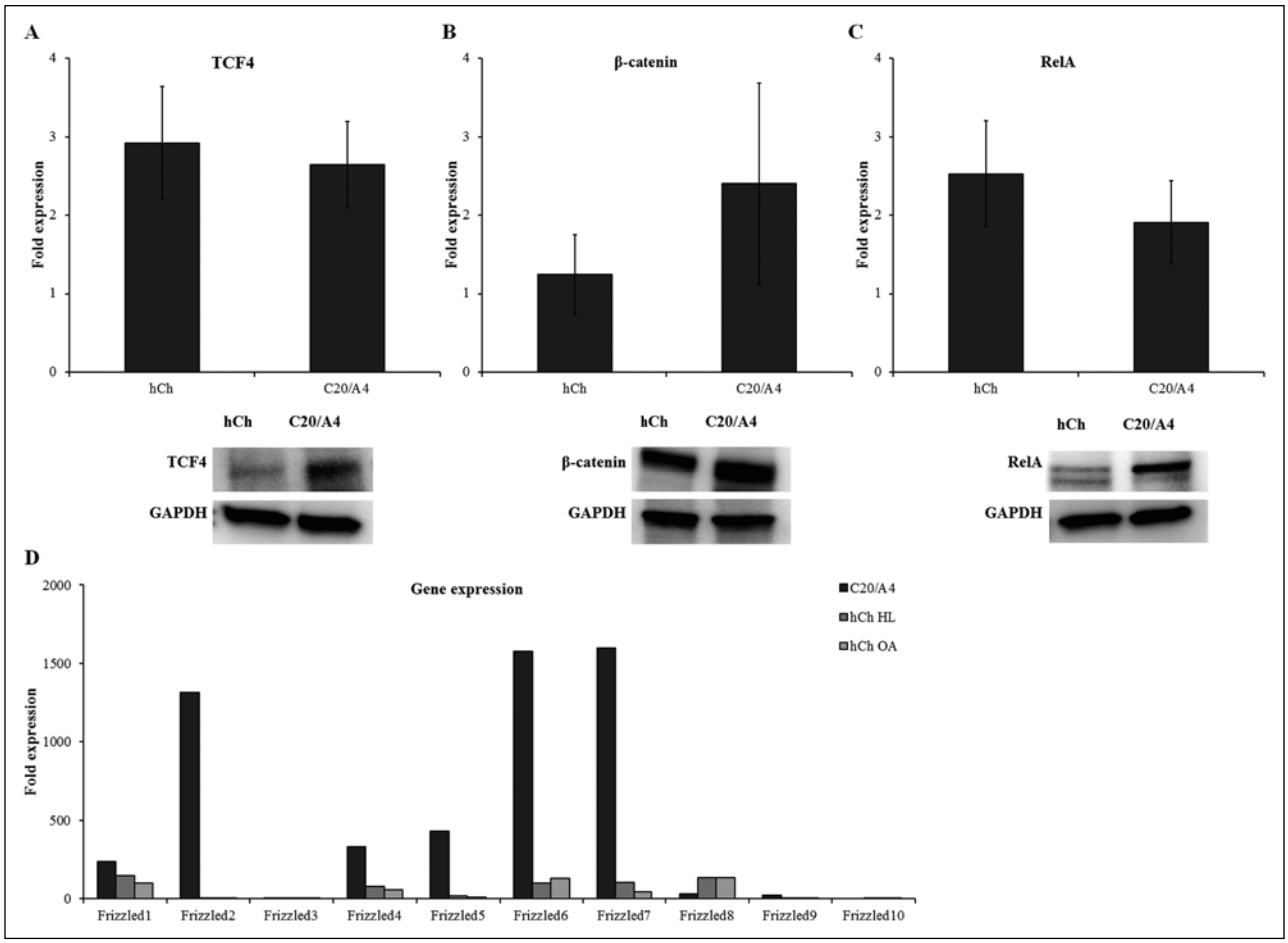

Figure 4. (**A**) Human primary articular chondrocytes (hCh) expressed significantly lower mRNA and protein levels of TCF4 compared with C20/A4 under basal conditions. (**B**) Slightly higher protein expression of β-catenin was found in C20/A4, compared with hCh. (**C**) No significant difference was found in mRNA expression of *RELA* between hCh and C20/A4, whereas protein expression was significantly higher in C20/A4. (**D**) *FRZD2, FRZD4, FRZD5, FRZD6* and *FRZD7* were significantly higher expressed in C20/A4 when compared to hCh. No differential expression was found between hCh from healthy looking regions (HL) and arthritic regions (OA) from the same joint. Gene expression levels were determined by qPCR after normalization for a house hold gene. Data are expressed as the mean of 3 independent experiments ± 95% confidence interval.

Primary Chondrocytes Express Lower Levels of TCF4 and RelA Compared To C20/A4

We did not find significant differences in mRNA expression of *TCF4,* β-*catenin*, or *RELA* between hCh and C20/A4. Even though the mRNA expression of *TCF4* seemed lower in C20/A4, protein levels were higher than in hCh (**Fig. 4A**). Both mRNA and protein expression of β-catenin were higher in C20/A4 when compared to hCh (**Fig. 4B**). As for RELA, mRNA expression was reduced in C20/A4 cells in comparison with hCh, whereas protein levels were higher in C20/A4 compared to hCh (**Fig. 4C**).

Finally, we studied the expression of several Frizzled receptors, in hCh from healthy looking and arthritic regions of the joint and C20/A4. We found significantly higher expression of *FRZ2, FRZ4, FRZ5, FRZ6*, and *FRZ7* in C20/A4 when compared to hCh (**Fig. 4D**). No differential expression of Frizzled receptors was found between hCh from healthy looking regions and arthritic regions from the same joint.

Discussion

Previously, our group reported the inhibitory effect of β-catenin on MMP expression in human chondrocytes by counteracting NFκB signaling.[8] This was in contrast to the observation that WNT/β-catenin signaling had a pro-catabolic effect by mediating NFκB-induced MMP expression in mouse chondrocytes.[7,13] These results suggested differential crosstalk between signaling pathways involved in regulation of MMP expression in chondrocytes. However, β-catenin was found to inhibit NFκB signaling in colon and

breast tumors in mice[11] and overexpression of β-catenin blocked NFκB signaling in various colon and liver cancer cells.[12] The discrepancy in the effect of β-catenin on NFκB-induced MMP expression between mouse chondrocytes and mouse cancer cells, suggests a differential role that is cell type specific rather than species specific. These findings indicate that the crosstalk between the WNT/β-catenin pathway and NFκB signaling might be dependent on intracellular and/or extracellular environment.

In this study, we provide evidence that the crosstalk between WNT signaling and NFκB might be cell type dependent in human as well. We found differential effects of WNT induction by recombinant WNT3A or the GSK3β inhibitor BIO and WNT inhibition by PKF115-584 on IL1β-induced MMP expression between hCh and C20/A4. These results are in line with previous findings on the role of WNT/β-catenin signaling in IL1β-induced MMP expression.[18,19] The consistent effect of induction as well as inhibition of WNT signaling to block IL1β-induced MMP expression might be due to TCF4-mediated upregulation of MMP expression through interaction with the NFκB pathway by binding RELA/p65.[14] WNT3A and BIO are both inducers of the WNT/β-catenin signaling pathway, resulting in high levels of β-catenin, which translocates to the nucleus to bind TCF4. As a result, TCF4 is no longer available to interact with the NFκB pathway. The inhibitory effect of the WNT/β-catenin inhibitor PKF115-584 might be because of its established disturbing action on the binding of β-catenin to TCF4. Similarly, PKF115-584 may also block the binding of TCF4 to RELA.[17,20] Stimulation of the WNT pathway by WNT3A and BIO might result in higher cytoplasmic levels of β-catenin, which inhibits NFκB signaling by binding RELA in chondrocytes and other cell types.[8,11,12] Moreover, PKF115-584 can exert the same effect by blocking the binding of β-catenin to TCF4, resulting in more β-catenin to be available for binding RELA. In addition, the different approaches of these activators might induce differential interaction with NFκB signaling. PKF115-584 might prohibit interaction of β-catenin with NFκB mediators by blocking the epitopes that are responsible for these interactions.

In hCh, knockdown of RELA induced TCF/LEF reporter activity by BIO. This is in line with the inhibitory effect of overexpression of RELA on β-catenin-mediated transcription, as described previously.[21] In contrast, reporter activity was inhibited in the absence of RELA in C20/A4. Additionally, the NFκB reporter in hCh is activated by overexpression of TCF4, in line with previous findings.[8] In contrast, we found a stimulating effect on NFκB reporter activity by BIO. This effect cannot be explained by the increased cytoplasmic levels of β-catenin, which might be available for binding RELA and subsequently blocking NFκB signaling.[11,12] In contrast to our findings in hCh, overexpression of TCF4 inhibited IL1β-induced NFκB reporter activation in C20/A4, indicating differential interference of RELA and TCF4 with the WNT pathway and NFκB signaling between cell types.

The inhibition of IL1β-induced *SERPINA1* mRNA expression by BIO and PKF115-584 in hCh and by PKF115-584 in C20/A4, provides further evidence for inhibition of MMP expression by WNT modulation through blocking of the NFκB signaling pathway. In C20/A4, PKF115-584 affected *SERPINA1* expression in accordance with the inhibitory effect on MMP expression. Stimulation of WNT signaling by WNT3A and BIO did not consistently affect NFκB signaling and/or its effect on MMP expression in C20/A4.

In search for clarification of discrepancies between hCh and C20/A4, we assessed the mRNA and protein expression of the major mediators in the WNT and NFκB signaling pathways. The higher protein expression of TCF4 in C20/A4 as compared to hCh indicates the insensitivity of C20/A4 to modulation of TCF4 by compounds or adenoviral overexpression of TCF4. Higher basal levels of TCF4 might lower the cells responsiveness to small changes, as shown in cancer cells in which NFκB signaling is constitutively active.[22] Lower sensitivity of the cells to changes in NFκB activity might be responsible for the small NFκB response of the C20/A4 to WNT modulation, as compared with the response of hCh to WNT modulation. Furthermore, higher protein expression of RELA in C20/A4 compared with hCh could cause the more abundant response on IL1β-induced NFκB reporter activity.

We found higher mRNA expression of *FRZ2, FRZ4, FRZ5, FRZ6*, and *FRZ7* in C20/A4, compared to hCh. Even though it is unknown which WNT ligands interact with these receptors, Frizzled5, Frizzled6 and Frizzled7 were identified as activators of canonical WNT/β-catenin signaling.[23-25] The differing receptor expression profiles may explain differences in biological responses on stimulation by WNT ligands. Differences in intracellular components of the WNT and NFκB signaling pathways may further contribute to the observed differences between hCh and the C20/A4 cell line.

It has been described that chondrocytes cultured in monolayers do not behave similarly to chondrocytes in their natural environment.[26,27] Chondrocytes dedifferentiate when cultured in monolayers and alter their gene expression profile.[28] Extending these findings to the immortalized cell line C20/A4 indicates that these cells might have dedifferentiated more than the primary chondrocytes used in this study. This could explain the differences that were found. In addition, these cells might resemble primary chondrocytes more when cultured in micromasses, as was found for the immortalized chondrocyte cell line C28/I2.[29] Micromass culture changes the microenvironment of the cells, and might therefore alter the differences between hCh and C20/A4 that we found in this study.

In conclusion, our findings indicate that distinct effects of WNT modulation on IL1β-induced MMP expression in hCh and C20/A4 might be caused by impaired sensitivity of C20/A4 to changes in NFκB signaling resulting from differences in basal expression of major mediators of the WNT pathway and NFκB signaling. Since we used primary articular chondrocytes and immortalized costal chondrocytes, dissimilarities found, might be due either to location of the body where these cells were derived from or different circumstances in which they were maintained. Either way, differences in responses are considered cell type specific. Therefore, precautions have to be taken when extending results on molecular interaction between WNT and NFκB signaling from cell lines, to primary chondrocytes. Based on this study, we conclude that the difference between animal and human chondrocytes in effects of WNT/β-catenin signaling on IL1β-induced MMP expression are likely caused by environmental factors like expression of key components in either signaling pathway rather than true species differences.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

This study was approved by our institutional review board.

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